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# *CERTIFICATION*

**Date:** July 2, 1998

**Re:** German Medical Patent 0 403 960 A2

To Whom it May Concern:

On this date our firm translated the attached material from German into English. It is a true and accurate translation to the best of our ability. If there are any questions, please contact our agency.

Sincerely,

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(19) European Patent Office (11) Publication Number: 0 403 960 A2

(12) European Patent Application

(21) Application Number: 90111263.1 (51) Int. Cl.5: A61K 47/48, C07D 213/52, C07C 321/08

(22) Date of Application: 14 June 1990

(30) Priority: 19 June 1989 Germany 3919923

(43) Publication Date of the Application:
27 December 1990 patent sheet 90/52

(84) States where Treaty is valid:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) Magnetic Protein Conjugate, Procedure for its Production and its Use.

(57) The invention concerns magnetic protein conjugates of the general formula I,

$$\text{M-NH-CO-}(CH_2)_n\text{-S-SP} \quad \text{I}$$

where n = 1-6, preferably where n = 2 or 3, where M is a dispersible, magnetically reacting material or particle that bears amino groups, and P is a protein. The invention concerns a procedure for its production as well as its use for the specific removal of cells or soluble antigens, receptors, substrates, cofactors or carbohydrate determinants from aqueous saline solutions or from body fluids, or within the framework of a diagnostic procedure or as a diagnostic method, preferably for the removal of cells, especially for bone marrow depletion or for HLA typing.

EP 0 403 960 A2

Magnetic Protein Conjugate, Procedure for its Production and its Use

The invention concerns magnetic protein conjugates of formula I,
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}S\text{-}SP$ I
where n = 1-6, preferably where n = 2 or 3, where

M is a dispersible, magnetically reacting material or particle that bears amino groups, and P is a protein.

P can be a protein in which the thiol group necessary for bonding in I is either present in the natural state or is produced by the reduction of disulfide bonds, or is added by chemical reaction.

In particular, P is an immunoglobulin or an immunoglobulin residue, preferably a monoclonal antibody or a Fab-, Fab'- or $F(ab)_2$ fragment, an antigen or an enzyme, hormone, lectin or growth factor residue.

P is preferably a monoclonal antibody of the IgG or IgM class, especially a monoclonal antibody which is targeted against an antigen, which is present in aqueous saline solutions or body fluids in soluble form, or a monoclonal antibody which is targeted against an antigen, which is expressed on cells, whereby the cells expressing the antigen can especially be cells of the myeloid or lymphatic system, cells of the peripheral blood, especially B-lymphocytes, T-lymphocytes or their precursor cells, or tumor cells, especially tumor cells of the bone marrow. Such cells can also be erythrocytes, bacteria, mycoplasms, or protozoan. Within the framework of the invention, viruses are also considered to be cells.

M is preferably a dispersible particle with a metal oxide core and an amino group-bearing hull coat, whereby the metal oxide core can include a group of paramagnetic substances, preferably a particle, the diameter of which lies between approximately 0.1 microns and approximately 100 microns, but preferably between approximately 0.1 microns and 1.5 microns.

The invention also concerns a procedure for the production of a magnetic protein conjugate of the general formula I, as well as the use of a conjugate of the formula I for the specific removal of cells or soluble antigens, receptors, substrates, co-factors or carbohydrate determinants from aqueous saline solutions or body fluids, as well as the use within the framework of a diagnostic technique or as a diagnostic technique, or use for bone marrow depletion or for HLA typing.

A bone marrow transplant is often the only possibility of treatment for some disorders, such as certain forms of leukemia and panmyelopathy (bone marrow shrinkage). With leukemias and certain lymphoid neoplasms, the patient is subjected to an entire body irradiation with an extremely high dose, and/or an aggressive chemotherapy. With such a treatment, the normal stem cells of the bone marrow, the precursors of all blood cells, are completely destroyed. The patient is therefore given the bone marrow of an appropriate donor, and the cells colonize the bone marrow space of the recipient, and thereby make possible the new development of the blood formation and the immune systems.
. This procedure is called allogene bone marrow transplantation.
Among other things, the T-lymphocytes of the donor, re-introduced with the transferred bone marrow, are responsible for the high risk of the allogene bone marrow transplant, since they recognize the cells of the recipient as foreign, and therefore attack and destroy them. This bone marrow rejection, which is often life-threatening for the patient, is called "graft-versus-host" reaction or "graft-versus-host" disease (GVHD). The risks of this "graft-versus-host" disease can be reduced by re-infusion of the patient with typed bone marrow of especially suitable donors, mostly from the family circle, if possible. The risk can also be reduced by identifying undesired cell populations, such as T-lymphocytes of the donor bone marrow, and by selectively eliminating them before the re-infusion into the patient. This elimination of donor T-cells can, for example, occur through selective lysis of the cells to be removed in the presence of complement, or through selective killing of the T-cells with the help of so-called immune toxins, or through another procedure, for example, through magnetic cell depletion of the bone marrow.

A cell depletion of the bone marrow of this type can be completed in a relatively simple manner, in such a way that the bone marrow is incubated with a monoclonal antibody from a mouse, which, for example, is specifically targeted against the T-cells of the bone marrow and therefore binds only to the T-cells. This type of T-cells covered



with monoclonal antibodies from the mouse can only be removed in a second step, in that they are incubated, for example with anti-mouse immunoglobulin from rabbits, which is bound to magnetic particles, whereby the T-lymphocytes are covered in a specific manner with the magnetic material, so that they can be removed from the bone marrow with the help of a magnet (see also Vartdal et al., Transplantation (1987), 43, 366-371 and the literature cited there).

In an analogous manner, other cell populations, such as tumor cells, can be removed from the bone marrow, which is significant for the so-called autologous bone marrow transplantation (see also Kvalheim et al., Cancer Research (19876), 47, 846-851 a, and the literature cited therein). As described by Kvalheim et al., ibid., the tumor cell-recognizing monoclonal antibody can thereby also be directly bound to the magnetic particle, so that the second antibody (rabbit anti-mouse), mentioned above, is no longer necessary.

The procedure described above for bone marrow depletion with the aid of monoclonal or polyclonal antibodies, which are bound to magnetic particles, is still very new and requires further development and experimentation. Magnetic particles that are suitable for this purpose can currently be purchased in various forms, and their production was described in more detail in the patent literature (for example, see Chagnon et al., EP 0125995 A2 (Priority US 493991 from 12 May 1983), Advanced Magnetics, or Ughelstad et al., WO 8303920 from 10 November 1983, SINTEF). It is known that these magnetic particles consist of a metal oxide core, in which paramagnetic substances can be included, and the core is surrounded by a hull coat, which can carry reactive groups such as aminophenyl-, amino-, carboxyl-, hydroxyl- or sulfhydryl- groups, which can be used for protein coupling (Chagnon et al., EP 0125995 A2).

For example, it is known that particles bearing carboxyl groups can be made to react with amino groups of proteins in the presence of a condensing substance (Chagnon et al., EP 0125995 A2).

The coupling of proteins on amino group-bearing magnetic particles with the use of glutaraldehyde is also known, whereby the coupling occurs across the amino groups (Chagnon et al., EP 0125985 A2).

It is also known that the particles bearing hydroxyl groups can be activated through reaction with p-toluolsulfonyl chloride, and that particles activated in such a way can be made to react with amino groups of proteins (Kvalheim et al., Cancer Research (1987), 47, 846-851).

All these coupling procedures have in common the fact that the protein is attached to the particle by means of its free amino groups. However, a coupling of this kind across amino groups can be a significant disadvantage with monoclonal antibodies, because the specificity and the reactivity of the antibodies is thereby impaired. This is a consequence of the fact that the amino groups, when in conjunction with an antibody, can be said to statistically divide themselves over the entire molecule and therefore are also localized in the antigen binding site of the Fab fragments, which causes a loss of specificity with a coupling across this amino group.
It is also known that antibodies, even without chemical linkage, can be pulled onto magnetic particles by mere adsorption, if the particles consist of a styrol-divinylbenzol co-polymerizate, which contains iron oxide , since protein is known to bind nonspecifically to polystyrol.

However, in this procedure, an impairment of the antibody specificity must also be anticipated. A further serious disadvantage of this procedure is that antibodies bound only by adsorption can be released again during the bone marrow depletion, and can thereby be passed on in the re-infusion of the depleted bone marrow into the patient, which can lead to serious side effects, especially if a previous therapy has been attempted with monoclonal

antibodies. However, this problem is known, and should be overcome through covalent linkage of the antibody to the magnetic particle.

It is also known that magnetic particles on polystyrol bases have the serious disadvantage of tending to aggregate and to taken up nonspecifically by cells.

Proceeding from this state of technology, it is the assignment of the following invention to develop a method by which monoclonal antibodies are coupled to magnetic particles) a) covalently and b) not across their amino groups. The purpose of the following invention is therefore, in other words, to find a coupling procedure in which the antigen binding site of the antibody is not altered, or the coupling of the antibody occurs away from the antigen binding site.
This purpose, according to the invention, is accomplished through the production of magnetic protein conjugates of the formula I.

It was already suggested that amino group-bearing magnetic particles can be transferred to magnetic particles which carry maleimido functions as their reactive group, and to conjugate these with proteins that possess thiol groups, whereby the thiol groups are either already present or are added by chemical means, or can be produced through the reduction of already-present disulfide bonds.





It was found that BioMag(R) magnetic particles, which bear free amino groups on their surface, can be activated by replacement with 2-iminothiolan (2-It) in such a way that they can be covalently linked through mere incubation with antibodies to the same. Magnetic antibody conjugates produced in this way are new.

It was found that BioMag(R) magnetic particles, which bear free amino groups on their surface, can also be activated by replacement with N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and following reductive cleavage of the disulfide bonds by means of dithiothreitol or mercaptoethanol, so that they can be covalently linked to antibodies through mere incubation with the same. Magnetic antibody conjugates produced in this way are also new.

It was also found that said magnetic particles can be covalently linked to antibodies after replacement with SPDP through incubation with antibodies which carry free SH-groups, without needing to activate the magnetic particle that has previously been modified by the replacement with SPDP by means of dithiothreitol or mercaptoethanol. Magnetic antibody conjugates produced in this way are also new.

Surprisingly, it was found that the loading of the magnetic particle with antibody can be increased if any thiol groups remaining after the coupling step are saturated through reaction with N-ethyl-maleimide or iodacetamide. Surprisingly, the stability of the magnetic antibody conjugate produced thereby is increased.

Also, it was found that the disulfide bond produced between magnetic particle and antibody can be cleaved again by reduction with dithiothreitol or mercaptoethanol. This type of cleavable magnetic particle-antibody conjugate is also new, and two special advantages of the invention lie in the ability of the spacer to be cleaved, and in the variable spacer lengths:
1. The cleavable spacer makes possible a positive selection of those antigens or cells which are recognized by the antibody coupled to the magnetic particle; this occurs by simple magnetic separation. The magnetic particles can be cleaved from the depleted antigens or cells by a reductive mechanism, and can be removed with the aid of a magnet.
2. The variable spacer length allows the variation of the distance between antibodies and magnetic particles within certain limits, and allows adjustment for the current coupling, separation or depletion problem. The selection capability is primarily a special advantage for the use of particles of different sizes, or during coupling of antibodies of different classes or isotypes.

Surprisingly, it was found that the specificity and reactivity of the antibodies coupled across disulfide bonds, or across the previously described spacers to magnetic particles, was retained, because the antigen binding site of the antibody is not altered or impaired by the binding of the antibody across its hinge region. Herein lies a great advantage of the invention as compared to the previously known coupling procedures, in which the antibodies, as previously described, are added on either through mere adsorption, or by the reaction of their amino groups with magnetic particles, which can impair both the specificity and the reactivity of the conjugated antibody. Compared to the adsorptive coupling, this also has the advantage of the antibody being chemically bound to the magnetic particle.

It was also found that the magnetic antibody conjugates, according to the invention, prove themselves to be especially advantageous because of their high specificity, for example, in the depletion of bone marrow. It was further found that the magnetic antibody conjugate according to the invention has also been shown to be advantageous within the framework of a diagnostic procedure or as a diagnostic technique, especially for example for HLA typing , because of its high specificity.

It was especially found that the magnetic antibody conjugate according to the invention is suitable for positive selection of antigens or cells, because the magnetic particles can be separated, after reductive cleavage of the disulfide bond of the spacer, from the isolated antigens or cells, with the aid of a magnet or centrifuge.

The production of magnetic antibody conjugates according to the invention is as follows for various monoclonal antibodies, which are targeted against cells of the bone marrow as well as for a polyclonal immunoglobulin of a rabbit described as an example; however, the said examples do not limit the invention. Furthermore, the use of the magnetic antibody conjugate, produced according to the example for the depletion of cells of the bone marrow, is also described as an example, without limiting the use to the named examples. The reductive cleavage of the disulfide bond of the spacers by means of incubation with dithiothreitol is also described as an example, without limiting the invention thereby.





Procedure for the production of magnetic protein conjugate of the formula I

a) amino group-bearing magnetic particles M are placed in a suitable solvent with a compound with the formula II, which reacts with amino groups
(diagram) II

where n = 1-6, under formation of an amide bond to a compound of formula III
(diagram) III
and this is added through reductive cleavage of the disulfide bond to a compound of formula IV
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}SH$ IV
which is finally placed in a suitable aqueous, salt-containing solvent, which does not denature proteins, such as, for example, physiological saline solution or a phosphate buffered saline solution, with a protein P that possesses a disulfide bond , such as an antibody, which is added to a compound of formula I, or

b) amino group-bearing particles M are reacted with a compound of formula IV, in which n = 3, as described above, in a suitable solvent with iminothiolan, whereupon the reaction of this compound of formula IV occurs with a protein P that possesses a disulfide bond , in the manner previously described, or

c) Amino group-bearing particles M are reacted as previously described with a compound of formula III, which is reacted in a suitable aqueous salt-containing solvent, which does not denature proteins, with a protein P that bears thiol group, such as a reduced antibody or a Fab or Fab' fragment that has reacted with a compound of formula I, whereupon the bond between protein P and spaced magnetic particles is stabilized by the addition of a suitable maleimido derivative, for example, N-ethyl-maleimide, or through the addition of iodacetamide or bromacetamide.

Suitable solvents for the coupling of a compound of formula II or of iminothiolan to magnetic particles must be made in such a way that the magnetic particles being used for coupling are not impaired in their physical and magnetic characteristics, especially in their size, dispersability, and surface composition by the solvent used. For magnetic particles, as they are described, for example in EP 0125995 A2 or WO 8303920, a solvent such as a mixture of water and dimethylformamide was found to be suitable.

Determination of the degree of coupling (micrograms antibody/mg iron)

Iron was determined through the use of atomic adsorption and nitrogen according to the Kjeldahl method. The values for the coupled protein-nitrogen were calculated according to the formula

micrograms P-N/ mg Fe = micrograms Tot-N/ mg Fe (experimental) - micrograms Tot-N/ mg Fe (control)

whereby the terms have the following meaning:
P-N: Protein-Nitrogen
Tot-N: total Nitrogen
Fe: iron



The quantities of protein bound to the particle (micrograms protein/ mg iron) were obtained from the protein nitrogen quantity for each mg iron by multiplication by a factor of 6.25. The calculated coupling rates are summarized in Table 1.

Procedure for the Depletion of Cells

A suspension of a cell mixture to be depleted in a salt-containing, preferably physiological aqueous solution or in body fluids is incubated with a compound of formula I at a suitable temperature between, for example, 0 Degrees C and 40 degrees C, preferably under agitation, and also preferably under sterile conditions, over a suitable period of time, and afterwards the magnetic particles are separated from the solution with a suitable magnet.

Suitable temperatures are, for example, 0 degrees C, room temperature, or 37 degrees C, but room temperature is preferred. The duration of the incubation depends on the incubation temperature and on the binding reactivity of the antibody and can, for example, be anywhere from a few minutes, up to 2 hours. A preferred time is 10 to 20 minutes of incubation at room temperature.

Procedure for the Isolation of Soluble Bio-Organic Molecules

This procedure basically follows the procedure for the depletion of cells.

Examples

The following examples serve for the more detailed explanation of the invention, but do not limit the invention. Magnetic particles, which were brought to reaction in the described manner with monoclonal antibodies, will be called "magnetobeads" from this point on, whereby their specificity is given with the antibody name.

Example 1

Production of a compound of formula IV, in which n = 3,
3 x 300 microliters of a commercially available suspension of the magnetic particle (BioMag(R), Advanced Magnetics) were each washed 3 x each with 10 ml phosphate-buffered saline solution pH 7.2 (PBS) and each was resuspended in 2 ml PBS. Afterwards, the addition of a solution of 2-iminothiolan-hydrochloride (2-It-HCl) in PBS occurred:
a) addition of 10 mg 2-It HCl in 0.5 ml PBS

b) addition of 2.5 mg 2-It HCl in 0.5 ml PBS
c) addition of 0.6 mg 2-Il HCl in 0.5 ml PBS

These suspensions were agitated for 1 hour at room temperature. Then the particles were centrifuged at 3000 x g and each was washed with 10 ml PBS.

Example 2

The production of a compound of formula III, in which n = 2,
3 x 300 microliters of a commercially available suspension of the magnetic particle (BioMag(R), Advanced Magnetics) were each washed 3 x each with 10 ml pH 7.2 PBS and each was resuspended in 3ml PBS. Then a solution of 5 mg N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) was reacted in 2 ml dry dimethylformamide (DMF) and agitated for 1 hour at room temperature. Then the particles were centrifuged at 3000 x g and each was washed with 10 ml PBS.





Example 3

Production of a compound of formula IV, in which n = 2

Three particle aliquots of formula III, produced according to example 2, were each resuspended in 2.4 ml PBS, each with 5 mg dithiothreitol , dissolved in 0.1 ml PBS, transferred and incubated at room temperature for 30 minutes under light agitation. Then the particles were centrifuged at 3000 x g and each was washed 3 x with 10 ml PBS and immediately used for the coupling reaction.

Example 4

Coupling of polyclonal rabbit anti-mouse immunoglobulin (KAM) to a compound of formula IV according to example 1a, in which n = 3, with variation of the antibody quantity.
Four particle aliquots of formula IV, produced according to example 1a from 300 microliter aliquots of BioMag(R) were placed in :
a) 2. ml PBS
b) 2.4 ml PBS
c) 2.3 ml PBS
d) 2.2 ml PBS resuspended and the suspension replaced with the following amounts of KAM:
a) no addition
b) 0.5 mg KAM in 0.1 ml PBS
c) 1.0 ml KAM in 0.2 ml PBS
d) 1.5 mg KAM in 0.3 ml PBS
The mixtures (each 2.5 ml) were each incubated at room temperature for 1 hour under agitation. Then the particles were centrifuged off at 3000 x g, washed 3 x each time with 10 ml PBS, resuspended in 5 ml PBS pH 7.2, sterilized by x-ray irradiation, and stored at 4 degrees C. The analytical data are summarized in Table 1.

Example 5

Coupling of the monoclonal antibody BMA 0110 (anti-CD2; IgG2b) to a compound of the formula IV according to example 1a, in which n = 3, with variation of the antibody quantity.
The coupling succeeded analogous to example 4, whereby the particle suspensions a-d of formula IV were replaced with the following amounts of BMA 0110:
a) no addition
b) 0.5 mg BMA 0110 in 0.1 ml PBS
c) 1.0 mg BMA 0110 in 0.2 ml PBS

d) 1.5 mg BMA 0110 in 0.3 ml PBS
Further processing succeeded analogous to example 4; the analytical data are listed in Table 1.

Example 6

Coupling of the monoclonal antibody BMA 0110 (anti-CD2; IgG2b) to a compound of the formula IV according to example 1a, in which n = 3, with variation of the antibody quantity.
The coupling succeeded analogous to example 5, whereby one of the compounds of formula IV is used according to example 1b (instead of example 1a).

Example 7

Coupling of monoclonal antibody BMA 030 (anti-CD3; IgG2b) to a compound of formula IV according to example 1a, 1b and 1c, in which n = 3, with the use of the same antibody concentrations.
Particle aliquots of formula IV produced According to example 1a, 1b and 1c were each resuspended in 2.0 ml PBS and each transferred with 0.5 ml of a solution of BMA 030 in PBS (corresponding to 1 mg BMA 030 in 0.5 ml PBS) and further processed analogous to example 4. The analytical data are summarized in Table 1.



Example 8

Coupling of monoclonal antibody BMA 033 (anti-CD3; IgG3) to a compound of formula IV according to example 1a, 1b and 1c, in which n = 3, by the use of the same antibody concentrations. The reaction succeeded analogous to example 7; the analytical data are listed in Table 1.

Example 9

Coupling of monoclonal antibody BMA 081 (anti-CD8; IgG2a) to a compound of formula IV according to example 1a, in which n = 3, with variation of the antibody quantities. The reaction succeeded analogous to example 5; the analytical data are listed in Table 1.

Example 10

Coupling of polyclonal rabbit anti-mouse immunoglobulin (KAM) to a compound of formula III according to example 2, in which n = 2, with variation of the antibody quantity.
A solution of 2 mg KAM in 0.4 ml PBS was replaced with 2 mg dithiothreitol and incubated at room temperature for 30 minutes. The reduced antibody isolated by means of filtration over Sephadex G25 in PBS pH 7.2 in an elution volume of 4.2 ml.
Particle aliquots of formula III, produced according to example 2, were resuspended as follows:
a) in 4.3 ml PBS
b) in 3.6 ml PBS
c) in 2.9 ml PBS
These suspensions were replaced with reduced antibodies as follows:
a) addition of 0.7 ml (approximately 0.3 mg protein)
b) addition of 1.4 ml (ca. 0.6 mg protein)
c) addition of 2.1 ml (ca. 0.9 mg protein)
The mixtures (each 5 ml) were each incubated at room temperature for 1 hour under agitation. Then the particles were centrifuged off at 3000 x g, washed 3 x each time with 10 ml PBS, resuspended in 5 ml PBS pH 7.2, sterilized by x-ray irradiation, and stored at 4 degrees C. The analytical data are summarized in Table 1.

Example 11

Tabelle 1

| Kopplungsgrade verschieden hergestellter Magnetobeads | | | | | | | |
|---|---|---|---|---|---|---|---|
| Beispiel | Antikörper | Isotyp | Spezifität | Kopplungsmethode | Kopplungsansatz | | |
| | | | | | Ak/Fe[1] (mg/mg) | Sp/Fe[2] (µmol/mg) | P/Fe[3] (µg/mg) |
| 4b | KAM | IgG | aMIg | 2-It/SS | 0.1 | 14.5 | 110 |
| 4c | KAM | IgG | aMIg | 2-It/SS | 0.2 | 14.5 | 173 |
| 4d | KAM | IgG | aMIg | 2-It/SS | 0.3 | 14.5 | 229 |
| 5b | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.1 | 14.5 | 107 |
| 5c | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.2 | 14.5 | 156 |
| 5d | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.3 | 14.5 | 178 |
| 6b | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.1 | 3.6 | 65 |
| 6c | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.2 | 3.6 | 78 |
| 6d | BMA 0110 | IgG2b | CD2 | 2-It/SS | 0.3 | 3.6 | 112 |
| 7a | BMA 030 | IgG2a | CD3 | 2-It/SS | 0.2 | 14.5 | 71 |
| 7b | BMA 030 | IgG2a | CD3 | 2-It/SS | 0.2 | 3.6 | 53 |
| 7c | BMA 030 | IgG2a | CD3 | 2-It/SS | 0.2 | 0.9 | 12 |
| 8a | BMA 033 | IgG3 | CD3 | 2-It/SS | 0.2 | 14.5 | 69 |
| 8b | BMA 033 | IgG3 | CD3 | 2-It/SS | 0.2 | 3.6 | 56 |
| 8c | BMA 033 | IgG3 | CD3 | 2-It/SS | 0.2 | 0.9 | 39 |
| 9b | BMA 081 | IgG2a | CD8 | 2-It/SS | 0.1 | 14.5 | 73 |
| 9c | BMA 081 | IgG2a | CD8 | 2-It/SS | 0.2 | 14.5 | 80 |
| 9d | BMA 081 | IgG2a | CD8 | 2-It/SS | 0.3 | 14.5 | 91 |
| 10a | KAM | IgG | aMIg | SPDP/SH | 0.06 | 3.2 | 29 |
| 10b | KAM | IgG | aMIg | SPDP/SH | 0.12 | 3.2 | 36 |
| 10c | KAM | IgG | aMIg | SPDP/SH | 0.18 | 3.2 | 55 |
| 11b | KAM | IgG | aMIg | SPDP/red | 0.1 | 3.2 | 27 |
| 11c | KAM | IgG | aMIg | SPDP/red | 0.2 | 3.2 | 51 |
| 11d | KAM | IgG | aMIg | SPDP/red | 0.3 | 3.2 | 74 |
| 12b | BMA 0110 | IgG2b | CD2 | SPDP/red | 0.1 | 3.2 | 27 |
| 12c | BMA 0110 | IgG2b | CD2 | SPDP/red | 0.2 | 3.2 | 47 |
| 12d | BMA 0110 | IgG2b | CD2 | SPDP/red | 0.3 | 3.2 | 57 |

Coupling of polyclonal rabbit anti-mouse immunoglobulin (KAM) to a compound of formula IV according to example 3, in which n = 2, with variation of the antibody quantity.
Four particle aliquots of formula IV, produced according to example 1a from 300 microliter aliquots of BioMag(R) were placed in :
a) 2.5 ml PBS
b) 2.4 ml PBS
c) 2.3 ml PBS
d) 2.2 ml PBS resuspended and the suspension replaced with the following amounts of KAM:
a) no addition
b) 0.5 mg KAM in 0.1 ml PBS
c) 1.0 ml KAM in 0.2 ml PBS
d) 1.5 mg KAM in 0.3 ml PBS
The mixtures (each 2.5 ml) were each incubated at room temperature for 1 hour under agitation. Then the particles were centrifuged off at 3000 x g, washed 3 x each time with 10 ml PBS, resuspended in 5 ml PBS pH 7.2, sterilized by x-ray irradiation, and stored at 4 degrees C. The analytical data is summarized in Table 1.

Example 12

Coupling of the monoclonal antibody BMA 0110 (anti-CD2; IgG2b) to a compound of formula IV according to example 3, in which n = 2, with variation of the antibody quantity. The coupling succeeded analogous to example 11; the analytical data are listed in Table 1.



Table 1

Coupling Degrees of differently produced Magnetobeads

| Example | Antibody | Isotype | Specificity | Coupling Method | Coupling Formulation | | |
|---|---|---|---|---|---|---|---|
| | | | | | Ab/Fe 1) (mg/mg) | Sp/Fe 2) (micromol/mg) | P/Fe 3) (micrograms/mg) |

(table)

1) quantity of antibody used per quantity of iron (mg/mg) in the current coupling formulation
2) quantity of spacer used per quantity of iron (micromol/mg) in the current coupling formulation
3) coupled protein quantity per iron (micrograms/mg)
KAM: rabbit anti-mouse immunoglobulin
CD: cluster of differentiation
aMIg: anti-mouse immunoglobulin

Claims

1. Magnetic protein conjugate of formula I,
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}S\text{-}S\text{-}P$ I
where n = 1-6, preferably where n = 2 or 3, where M is a dispersible, magnetically reacting material or particle that bears amino groups, and P is a protein.
2. Magnetic protein conjugate according to claim 1, characterized by the fact that
the thiol groups of the protein P are either present in the natural state or are produced by the reduction of disulfide bonds, or are added to the protein by chemical reaction.

3. Magnetic protein conjugate according to claim 1, characterized by the fact that
P is a polyclonal immunoglobulin.

4. Magnetic protein conjugate according to claim 1, characterized by the fact that
P is a monoclonal antibody or a Fab, Fab' or $F(ab)_2$ fragment.

5. Magnetic protein conjugate according to claim 1, characterized by the fact that
P is an antigen or an enzyme, hormone, lectin or growth factor residue.

6. Magnetic protein conjugate according to claim 4, characterized by the fact that
P represents a monoclonal antibody of the IgG or IgM class.

7. Magnetic protein conjugate according to claim 4, characterized by the fact that
P represents a monoclonal antibody which is targeted against an antigen, which is present in aqueous saline solutions or body fluids in soluble form.

8. Magnetic protein conjugate according to claim 4, characterized by the fact that
P represents a monoclonal antibody which is targeted against an antigen, which is expressed on cells, whereby the cells expressing the antigen can especially be cells of the myeloid or lymphatic system, cells of the peripheral blood, especially B-lymphocytes, T-lymphocytes or their precursor cells or tumor cells, especially tumor cells of the bone marrow.

9. Magnetic protein conjugate according to claim 4, characterized by the fact that
P represents a monoclonal antibody which is targeted against an antigen which is expressed on bacteria, mycoplasms, protozoan or viruses.

10. Magnetic protein conjugate according to claim 1, characterized by the fact that
P represents an antigen.

11. Magnetic protein conjugate according to claim 1, characterized by the fact that
M is a dispersible particle with a metal oxide core and an amino group-bearing hull coat, whereby the metal oxide core can include a group of paramagnetic substances.

12. Magnetic protein conjugate according to claim 11, characterized by the fact that
the diameter of the particle lies between approximately 0.1 microns and approximately 100 microns, but preferably between approximately 0.1 microns and 1.5 microns.

13. Compound of formula III
(diagram) III
wherein M has the meaning indicated in claim 1.

14. Compound of formula IV
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}SH$ IV
wherein M has the meaning indicated in claim 1.

15. Procedure for the production of magnetic protein conjugate of the formula I
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}S\text{-}SP$ I, characterized by the fact that
amino group-bearing magnetic particles M are reacted with a compound with the formula II, which reacts with amino groups
(diagram) II

where n = 1-6, under formation of an amide bond to a compound of formula III
(diagram) III

and this is added through reductive cleavage of the disulfide bond to a compound of formula IV
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}SH$ IV,
which is finally reacted with a protein P that possesses disulfide bonds to a compound of formula I.





16. Procedure for the production of a magnetic protein conjugate of formula I, characterized by the fact that amino group-bearing particles M are reacted with iminothiolan with a compound of formula IV
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}SH$ IV
in which n = 3, and the compound of formula IV is reacted with a protein P that possesses a disulfide bond to a compound of formula I.

17. Procedure for the production of a magnetic protein conjugate of formula I, characterized by the fact that amino group-bearing particles M, as in claim 15, are reacted with a compound of formula III, which is reacted with a protein P that bears thiol group attached to a compound of formula I.

18. Procedure for the production of a magnetic protein conjugate according to at least one of the claims 15, 16 or 17, characterized by the fact that
the bond between protein P and spaced magnetic particles is stabilized by the addition of an N-substituted maleimido compound, preferably through the addition of N-ethyl-maleimide, or through the addition of N-($C_1$-$C_6$-alkyl)-maleimide, especially through the addition of N- ethyl-maleimide, or through the addition of maleimidocarbonic acid, or through the addition of iodacetamide or bromacetamide.

19. Procedure for the removal of dissolved antigens, antibodies, receptors, substrates, co-factors, or of carbohydrate determinants from aqueous saline solutions or body fluids, characterized by the fact that
the solution is incubated with a suitable magnetic protein conjugate and the magnetic protein conjugate is separated by magnetic means after specific adsorption of the components to be removed, and the specifically adsorbed components are again separated from the magnetic protein conjugate, and cleaved through reduction of the disulfide bond of the spacer to the compound of formula I.

20. Procedure for the removal of cells from aqueous saline solutions or body fluids, characterized by the fact that the cell suspension is incubated with a suitable magnetic protein conjugate of formula I, and the magnetic protein conjugate is separated by magnetic means after specific adsorption of the cells to be removed, and the specifically adsorbed cells or particles are again released from the magnetic protein conjugate, or cleaved by the reduction of the disulfide bond of the spacer to the compound of formula I.

21. Procedure for the positive selection of soluble components according to claim 19, or of cells according to claim 20, characterized by the fact that
the disulfide bond of the spacer is cleaved reductively and the magnetic particle is removed by means of a magnet or a centrifuge.

22. Use of a magnetic protein conjugate according to claim 1 for the specific removal of cells or soluble antigens, receptors, substrates, co-factors or carbohydrate determinants from aqueous saline solutions or body fluids or use within the framework of a diagnostic procedure or as a diagnostic technique.



23. Use of a magnetic protein conjugate according to claim 1 for the removal of cells according to claim 8 or 9, especially for bone marrow depletion or for HLA typing.

24. Use of a magnetic protein conjugate according to claim 1 for the positive selection of cells or soluble antigens, receptors, substrates, co-factors or carbohydrate determinants, but especially for the positive selection of cells, especially for the positive selection of cells according to claim 8.

Patent Claims for the following Treaty States: ES, GR

1. Procedure for the production of a magnetic protein conjugate of formula I,
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}S\text{-}S\text{-}P$ I
characterized by the fact that
amino group-bearing magnetic particles M are reacted with a compound with the formula II, which reacts with amino groups
(diagram) II

where n = 1-6, under formation of an amide bond to a compound of the general formula III
(diagram) III
and this is added through reductive cleavage of the disulfide bond to a compound of formula IV
$M\text{-}NH\text{-}CO\text{-}(CH_2)_n\text{-}SH$ IV,
which is finally reacted with a protein P that possesses disulfide bonds to a compound of formula I.

2. Procedure according to claim 1, characterized by the fact that the protein used bears a thiol group.

3. Procedure according to claim 1, characterized by the fact that P is a polyclonal immunoglobulin.

4. Procedure according to claim 1, characterized by the fact that P is a monoclonal antibody, a Fab-, Fab'- or $F(ab)_2$ fragment, an antigen or an enzyme, hormone, lectin or growth factor residue.

5. Procedure according to claim 1, characterized by the fact that P represents a monoclonal antibody of the IgG or IgM class.

6. Procedure according to claim 1, characterized by the fact that protein conjugate according to claim 4, characterized by the fact that M is a dispersible particle with a metal oxide core and an amino group-bearing hull coat.

7. Procedure according to claim 1, characterized by the fact that
M is a dispersible particle with a metal oxide core and an amino group-bearing hull coat, and the diameter of the particle lies between approximately 0.1 microns and approximately 100 microns.